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TECHNICAL MANUSCRIPT 310

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UNITED STATES ARMY
BIOLOGICAL CENTER
FORT DETRICK

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AGENT DEVELOPMENT AND ENGINEERING LABORATORIES

Project 1C522301A059

July 1966

In conducting the research reported here, the investigators adhered to "Principles of Laboratory Animal Care" as established by the National Society for Medical Research.

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## ABSTRACT

Rats challenged with 16 or more units of anthrax toxin and stressed by holding at 4 C developed a hypothermia of 14 to 15 C and time to death was greatly increased. This is in contrast to other bacterial toxins and venoms. However, rats challenged with eight units of toxin died at 4 C but not at 24 C, and rats held at 36 C are more susceptible to toxin. Specific antiserum injected at 30, 45, 60, and 90 minutes after toxin challenge increased the time to death but resulted in survivors only at the lowest level of toxin, 15 units. When tested against a 30-unit challenge, caffeine, N-allylmorphine, ACTH, hydrocortisone, and pentobarbital sodium treatment did not result in any survivors. Although time to death was extended in certain cases, their interactions at 4 or 24 C were not of practical significance in extending time to death.

## I. INTRODUCTION

Anthrax toxin was recently shown by Lincoln et al.[1] to cause death by depression of the deep respiratory centers of the brain. During the early stages of intoxication, electrical cerebral cortical activity was depressed or silenced; however, specific anthrax antiserum rapidly restored that activity to normal. This observation is unique, as the effect of other toxins such as botulinum toxin and snake and spider venoms appear irreversible* and unaffected by atropine sulfate and pyridine -2- aldoxime methiodide. The fact that antiserum administered at 30-minute intervals for 4 to 8 hours restored normal cerebral cortical electrical activity suggests that other chemotherapeutic materials and treatments may be useful in treating the toxemic phase of anthrax.

Bacterial toxins have been shown to vary in toxicity with low ambient temperature of the test animal.[2,3] Cold also has been shown to influence the action of certain drugs in warm-blooded animals.[4-8] Recently, Stahnke[3] showed that both rattlesnake and scorpion venom increased in toxicity for the rat under cold stress or when epinephrine was administered alone or without stress. Similarly, with S. marcescens endotoxin[2] mice were more susceptible to the lethal effects of the toxin after cold exposure; however, cortical hormones effectively counteracted the increased toxicity of the toxin at 4 C and increased survival at room temperature. These apparent changes in toxicity were attributed to the physiological effects of stress rather than temperature per se.

We report here the effects of environmental temperature on the toxicity of anthrax toxin and the use of hypothermia combined with antiserum and various drugs as a chemotherapeutic means of treating the toxemic phase of the disease.

## II. MATERIALS AND METHODS

Anthrax toxin was prepared by the method described by Haines et al.[9] and concentrated by drying. An initial dilution to 400 units per ml was made with gel phosphate; further dilutions in saline were made as needed so that the intravenous (IV) challenge dose was contained in 1 ml. Anthrax toxin was quantitated with Fischer 344 white rats by the method described by Haines et al;[9] this was the rat used in these studies.

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* J.A. Vick, unpublished data.

The body temperature of rats challenged with toxin and held in environments of 4 ± 1 C, 24 ± 2 C (room temperature), and 38 ± 1 C was determined by a thermocouple needle* inserted 1 to 1.5 cm into the rear leg muscle. Temperature was read directly from a recorder** calibrated against a Bureau of Standards mercury thermometer.

Further treatments consisted of pharmacological doses given IV: ACTH, 10 units per rat; hydrocortisone, 10 mg per rat; pentobarbital sodium, 20 mg per rat, or specific anthrax antiserum, 0.5, 1.0, or 3.0 ml per rat. Each ml of antiserum neutralized in vitro about 7,000 rat units of toxin.

Body temperatures were measured in six groups of rats, three control groups and three challenged with lethal doses of anthrax toxin (Figure 1). One each of the challenged and of the control groups was maintained at 37, 24, or 4 C.

## III. RESULTS

The body temperature of rats held at 37 C was increased approximately 2.5 C in the first 10 minutes over that of animals held at 24 C. Temperature peaked at 4.0 C above normal, and then slowly adjusted at about 3.0 C above normal. At death, the body temperature of toxin-challenged rats, raised approximately 0.5 C over that of unchallenged control animals did not fall below that of the control. The harmonic mean time to death of 86 minutes was 35 minutes shorter than that of animals held at 24 C.

Throughout the entire 200-minute observation period the unchallenged rats at 24 C had a body temperature of 38 ± 1 C; the temperature of rats challenged with 27 units of toxin was the same as that of the controls for the first 70 minutes, then declined to about 36 C at a time to death of 121 minutes.

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* High Temperature Instrument Co., Philadelphia, Pa., Model HTI-AYP-1.00-CC-2, insulated junction, needle length 1.0 inch, gauge 23.

** Minneapolis Honeywell REG Company, Brown Instrument Division, Philadelphia, Pa., Model 153 x 65V4-X-S1, 4-probe recorder.

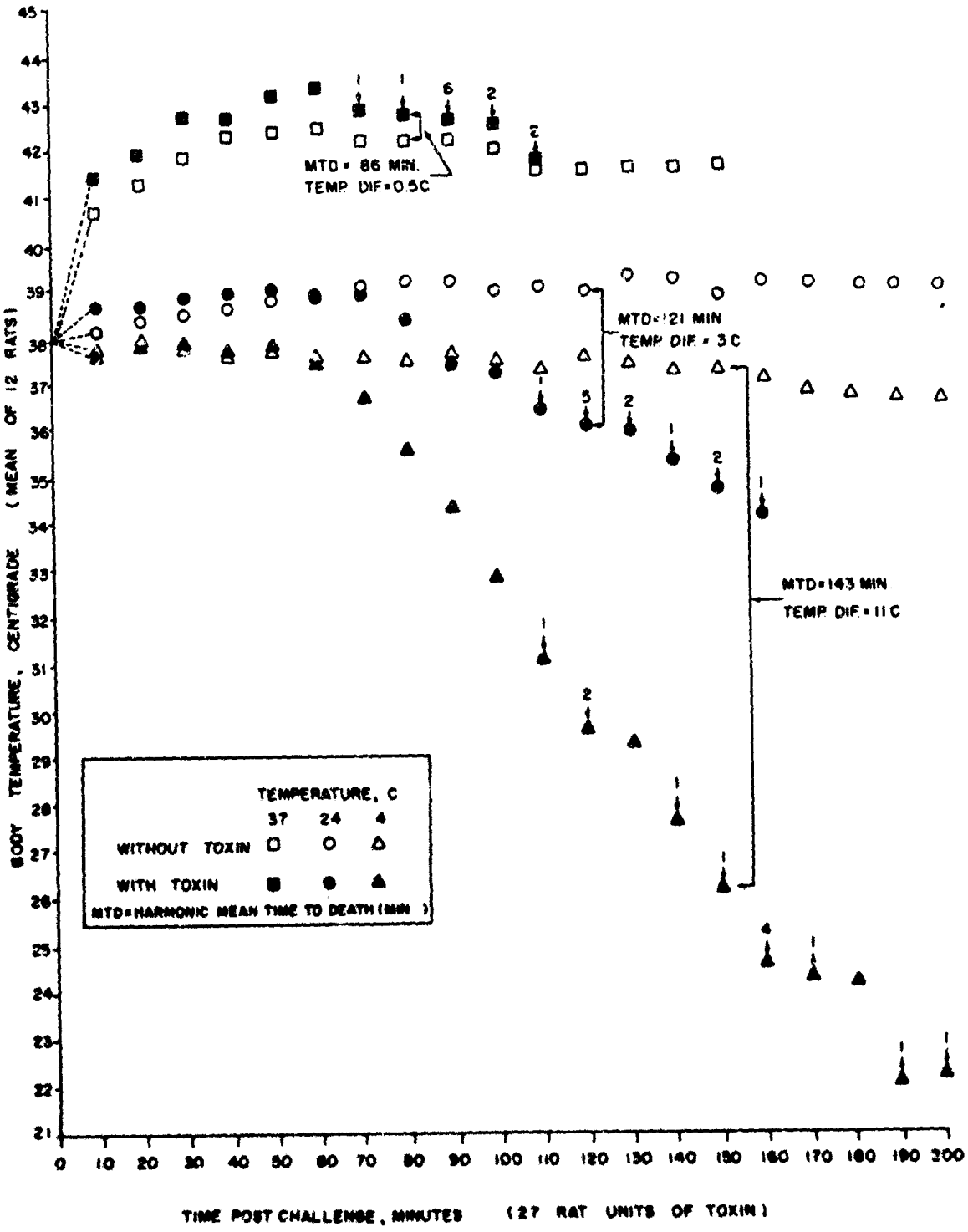


Figure 1. Time-Temperature Relationship of Rats Dying with a Lethal Dose of Anthrax Toxin (27 Units/Rat). Numbers 1 through 6 indicate the number of animals dying at indicated time ± 5 minutes. MTD, harmonic mean time to death.

The body temperature of unchallenged rats held at 4 C was 1 or 2 C lower than that of controls at room temperature. Toxin-challenged rats held at this ambient temperature maintained a body temperature similar to that of the control for 50 minutes, then body temperature rapidly declined. That of the first animal to die was 31 C; with death at 143 minutes, body temperature was 26 C, and the last animal to die had a body temperature of 24 C. Hypothermia is clearly evident after toxin challenge in animals maintained at 24 C, and it is more severe in animals housed at 4 C. These data indicate anthrax toxin is not pyrogenic, an observation made earlier by Smith et al.[10,11] on temperature and infection relationship of the disease in the spore-challenged guinea pig.

Further tests (Figure 2) with rats held at 4 and 24 C and given 4 to 60 units of toxin showed that cold-stressed rats died with significantly ($P<0.01$) extended time to death that was related to the toxin dose. The 8-unit dose of toxin (Figure 2A), however, was nonlethal at 24 C but lethal at 4 C. The 4-unit challenge dose was not lethal in these tests. At 37 C all animals died and time to death was significantly shorter than at 24 and 4 C (Figure 2B).

In spite of the increased susceptibility to toxin, it was possible that the increased time to death of animals stressed at 4 C might allow a longer time period during which treatment with specific antiserum would prevent death. This concept was tested in a factorially designed experiment in which three dosages of toxin, 15, 30 and 60 units, were administered to rats held at 4 and 24 C. Treatment with 0.5, 1.0, or 3.0 ml of specific equine antiserum was initiated at 30, 45, 60, or 90 minutes after toxin challenge. Results are given in Table 1. At toxin dosages of 30 and 60 units, no treatment resulted in survivors; however, at 15 units animals survived if antiserum were given at or prior to 60 minutes, but died with an extended time to death when the administration of antiserum was delayed until 90 minutes. Both stress at 4 C and antiserum extended time to death; however, there was no statistically significant interaction among any of the variables tested. Because survivors occurred in the 15-unit challenge dose, statistical analysis was limited to the 30- and 60-unit doses.

We tested the possible interaction of selected chemicals at pharmacological dosages and 4 C stress in extending time to death or survival of animals challenged with approximately 30 units of toxin (Table 2). Although the time to death was influenced, none of these treatments resulted in survival. Barbiturate, known to depress metabolism of the brain, extended the time to death at 24 C but shortened it at 4 C. The steroids, which are used to minimize the effects of stress, were essentially without effect. The central stem stimulants caffeine and D-allyl-morphine also had no practical effect. The interaction of barbiturates with hydrocortisone greatly extended the time to death at 24 C but shortened it at 4 C. Other interactions were too small to be considered of practical significance.

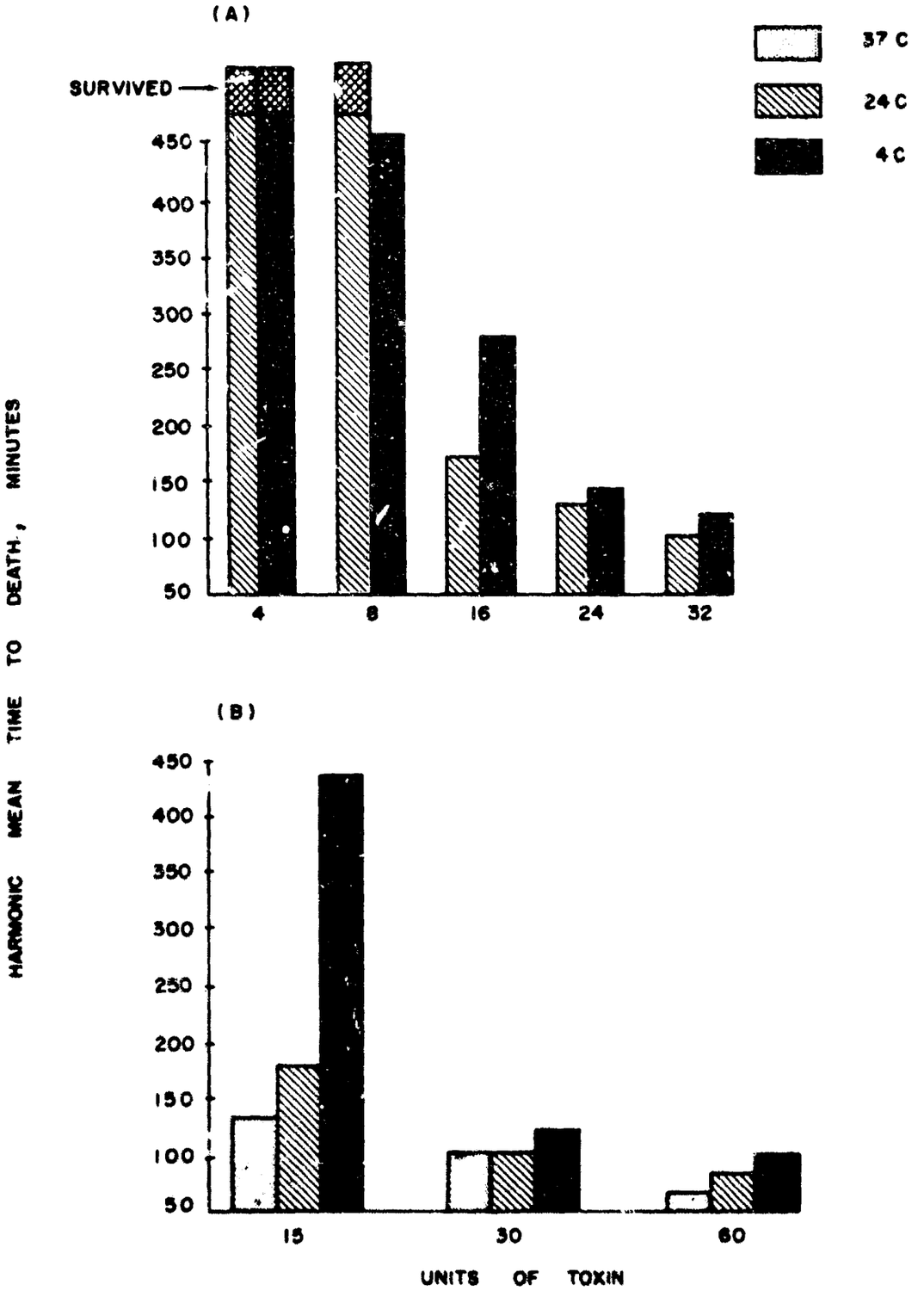


Figure 2. Harmonic Mean Time to Death for Six Rats Challenged with Anthrax Toxin, as Influenced by Temperature.
A. Challenged at 4 and 24 C.
B. Challenged at 4, 24, and 37 C.

TABLE 1. SURVIVAL TIME OF RATS CHALLENGED WITH ANTHRAX TOXIN AND TREATED WITH ANTISERUM

| Temp., C | Antiserum: Dosage, ml | Antiserum: Time Administered After Toxin, min | Harmonic Mean Time to Death, min[a] for Challenge with Indicated Units of Toxin: 15 | 30 | 60 |
|---|---|---|---|---|---|
| 24 (Room) | None[b] (Control) | | 233 | 100 | 89 |
| | 0.5 | 30 | S[c] | 217 | 72 |
| | | 45 | S | 122 | 89 |
| | | 60 | S | 101 | 72 |
| | | 90 | 322 | 95 | 84 |
| | 1.0 | 30 | S | 138 | 72 |
| | | 45 | S | 128 | 71 |
| | | 60 | S | 108 | 68 |
| | | 90 | 238 | 103 | 73 |
| | 3.0 | 30 | S | 340 | 88 |
| | | 45 | S | 139 | 79 |
| | | 60 | S | 126 | 75 |
| | | 90 | 269 | 109 | 83 |
| 4 | None (Control) | | 429 | 121 | 99 |
| | 0.5 | 30 | S | 369 | 93 |
| | | 45 | S | 138 | 83 |
| | | 60 | S | 134 | 83 |
| | | 90 | 501 | 81 | 96 |
| | 1.0 | 30 | S | 346 | 124 |
| | | 45 | S | 129 | 75 |
| | | 60 | S | 150 | 79 |
| | | 90 | 398 | 148 | 107 |
| | 3.0 | 30 | S | 609 | 224 |
| | | 45 | S | 199 | 83 |
| | | 60 | S | 164 | 85 |
| | | 90 | 573 | 188 | 75 |

a. Mean of six rats.
b. Control is mean of 18 rats.
c. Survived.

TABLE 2. MEAN TIME TO DEATH FOLLOWING ANTHRAX TOXIN CHALLENGE AS INFLUENCED BY INDICATED TREATMENTS

| Toxin, Units/Rat | Treatments | Harmonic Mean Times to Death, min[a]/ Temperature 24 C | 4 C | Difference |
|---|---|---|---|---|
| 27 | Barbiturate (B) | 190 | 142 | -48 |
| | Hydrocortisone (H) | 116 | 139 | +23 |
| | Adenocorticotropic Hormone (A) | 123 | 160 | +37 |
| | B + H | 221 | 131 | -90 |
| | B + A | 137 | 128 | - 9 |
| | Controls | 120 | 178 | +58 |
| 30 | Caffeine (C) | 98 | 113 | +15 |
| | D-Allyl-Morphine (M) | 105 | 121 | +16 |
| | Barbiturate (B) | 137 | 125 | -12 |
| | C + B | 126 | 127 | + 1 |
| | B + M | 120 | 133 | +13 |
| | Controls | 108 | 117 | + 9 |

a. Mean of six rats.

## IV. DISCUSSION

Our work has been directed toward finding ways to extend the time during which anthrax toxemia may be successfully treated. For the progressing septicemic disease, bactericidal antibiotics and antiserum used together resulted in greater survival than either material alone.[13] In this study, in which we considered only the toxemic phase of anthrax, we show that the time during which antiserum effectively influenced survival or the time to death is limited; only with 15 units of toxin, but not with 30 or 60 units, could death be prevented by administration of antiserum. It is evident that toxin is irreversibly fixed to

essential tissue sites soon after injection. Lincoln et al.[1] used 4- to 5-kg monkeys given 10,000 rat units of toxin, which resulted in death at 26 to 34 hours. However, they obtained two survivors of four animals tested if antiserum were given at 8 hours; all survived if antiserum were administered at 4 hours and none if given 10 hours after challenge.

Anthrax toxin is a complex toxin, composed of at least three components.[13] At least two components, lethal factor and protective antigen (LF and PA), are required for lethality. It seems probable that antiserum inactivates the LF component of the toxic complex, in that Molnar and Altenbern[14] have shown that the PA component disappears from the blood stream much more rapidly than the LF, and Lincoln et al.[1] showed that some component passed the blood-brain "barrier" to cause depression of the EEG in all animals within 1 minute and coma in 33% of the animals at 5 to 7 minutes. Later unpublished work indicates that PA is the component affecting the immediate EEG response and the interaction with LF that depresses the deep respiratory centers of the brain and leads to death. The LF component appears to be the slower of the essential components to be fixed onto tissue sites; therefore, treatment of anthrax should consider more specifically the LF component. In developing immunity, antigens used to produce antiserum should contain the LF component,[15] and strains used to immunize should be selected for strong LF production.

The cold stress (4 C) of animals challenged with anthrax toxin extended the time to death but made the host more susceptible to toxin; 8 units of toxin killed the 4 C stressed animals but not those held at 24 C. Molnar and Altenbern[14] decreased the lethality of the anthrax toxin by changing the ratio of PA to LF component. It seems possible that our results also may reflect a change in the ratio of components attached to tissue sites because hypothermia could be affecting the binding sites of PA, LF, or both to change the time to death.

Stressing animals challenged with anthrax toxin by holding them at 4 C greatly extended time to death; in contrast to snake venoms[3] and endotoxins,[2] however, anthrax toxin and endotoxin both resulted in hypothermia. The increased time to death by stress at 4 C did not increase the time during which chemotherapeutic treatment was effective, and none of the drugs tested extended the time to death in a practical sense. In spite of the negative results of these initial studies in either extending the time during which anthrax toxemia may be successfully treated or in finding an effective treatment, we have learned that anthrax toxin appears to be a unique pharmacological research material that could help in understanding and correcting respiratory depression of central origin as well as in other research problems.

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Rats challenged with 16 or more units of anthrax toxin and stressed by holding at 4 C developed a hypothermia of 14 to 15 C and time to death was greatly increased. This is in contrast to other bacterial toxins and venoms. However, rats challenged with eight units of toxin died at 4 C but not at 24 C, and rats held at 36 C are more susceptible to toxin. Specific antiserum injected at 30, 45, 60, and 90 minutes after toxin challenge increased the time to death but resulted in survivors only at the lowest level of toxin, 15 units. When tested against a 30-unit challenge, caffeine, N-allyl-morphine, ACTH, hydrocortisone, and pentobarbital sodium treatment did not result in any survivors. Although time to death was extended in certain cases, their interactions at 4 or 24 C were not of practical significance in extending time to death.

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